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SUPERNUMERARY LARVAL INSTARS IN CYCLORRHAPHOUS DIPTERA

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Larvae of cyclorrhaphous Diptera usually live in rapidly decaying media. They are well adapted to develop in these conditions by having a short feeding period, rapid growth rate, enormously high total metabolism, reduced number of larval instars, endomitotic growth of the larval tissues and presence of the puparium which protects the metamorphosis stages inside the sclerotized cuticle of the last larval instar. Consequently, the regulatory mechanisms for development, represented in insects by the neuroendocrine system, have undergone in this case certain modifications and deviations from the general scheme common to most other insects. For instance, in all groups of Exopterygote insects so far investigated, and also in many groups of Endopterygotes, it is possible to produce the supernumerary larval (or pupal) instars by transplanting active endocrine glands or applying juvenile hormone or ecdysone and their synthetic analogues (Pflugfelder, 1958; Novák, 1966; Sláma, 1971). However, it appears very difficult or impossible to obtain supernumerary larval instars in these cyclorrhaphous flies where such applications of the juvenile hormone resulted only in a delay of metamorphosis, or the formation of atypical puparia (Possompès, 1953), or in persistence of pupal characters in the adult (Srivastava and Gilbert, 1969; Ashburner, 1970). Ecdysone and its analogues, which have been most intensively studied and in fact isolated on the basis of the *Calliphora* assays, are merely known to cause puparium formation connected occasionally with an incomplete contraction of the puparium (Fraenkel, 1935; Butenandt and Karlson, 1954; Thomson and Horn, 1969), but no extra-larval ecdysis. Possible arguments for explaining the difficulties in producing supernumerary larval instars in this group traditionally focused on limitations of the ploidy in the endomitotic type of growth, or on the constant, and perhaps genetically fixed, number of larval instars.

Our approach to the problem of supernumerary instars in higher Diptera began with an analysis of the conditions required for obtaining similar effects in other insects. The most relevant for our study was the critical period after which both the juvenile hormone and ecdysone were unable to affect the developmental program in a given instar. The critical period for determination of the pupal or adult characters occurs soon after ecdysis, especially in rapidly developing species. It can generally be prolonged or postponed by factors like low temperature, starvation or inhibition of hormonal release (Sláma, 1971). For these reasons we have investigated conditions influencing the initiation of metamorphosis in the last larval instars of two species of flies.

Material and Methods

Larvae of the blowfly, *Calliphora vomitoria* (L.), and the fleshfly, *Sarcophaga argyrostoma* Robineau-Desvoidy, were bred on pork liver at 26° C. The freshly ecdysed larvae of the third instar were obtained from fully grown second instar larvae which were removed from the liver and placed on wet filter paper before ecdysis. The identification of separate larval instars was made according to the morphological criteria described by Possompés (1953). The starved larvae were kept on filter paper in Petri dishes containing a water vial plugged with cotton.

Ecdysterone (natural product isolated from *Polypodium vulgare* L. by Dr. J. Jizba) dissolved in a 10% ethanol solution was injected by means of finely drawn glass pipettes. The larvae were previously immobilized by chilling.

The material for histological examination was placed in Carnoy fixative, embedded in paraffin, and the sections were stained by Mallory's trichrome and Feulgen methods.

Results

All the experiments described were performed on *Calliphora* and most were repeated on *Sarcophaga*. The results were almost identical for both the species and thus we present the quantitative data only for one of them, *i.e.*, *Calliphora*.

1. *Effects of feeding on the initiation of development*

The blowfly larvae develop in three instars lasting approximately 18, 24, and 130 hours, respectively. They feed from hatching until the third day of the last instar, then leave the food and form puparia about 2 days later (Fig. 1).

It is generally known that feeding may stimulate the release of hormones regulating developmental cycles in larval instars and reproductive cycles in adults. At the beginning we have attempted to find out whether that was so in the case of third instar larvae of the blowfly. As is seen in the results in Figure 1, the larvae starved from the moment of last larval ecdysis survive for many days without any sign of further development. When such starved larvae are allowed to feed, they resume development leading to puparium formation. The timing of the developmental events is roughly the same as in normal third instar larvae, except that pupariation is delayed for a period corresponding to the period of starvation. Manifestly, the development leading to puparium formation is dependent on feeding after the last larval ecdysis.

In further experiments we determined the minimum amount of feeding necessary to initiate the developmental process. We found that as little as 3 to 10 hours of feeding could in certain cases lead to formation of small puparia from which emerge dwarf adults. Forms intermediate between larva and puparium were never observed. These findings suggest that the mechanisms for the metamorphosis process as a whole are irreversibly activated by a brief period of feeding after the last larval ecdysis.

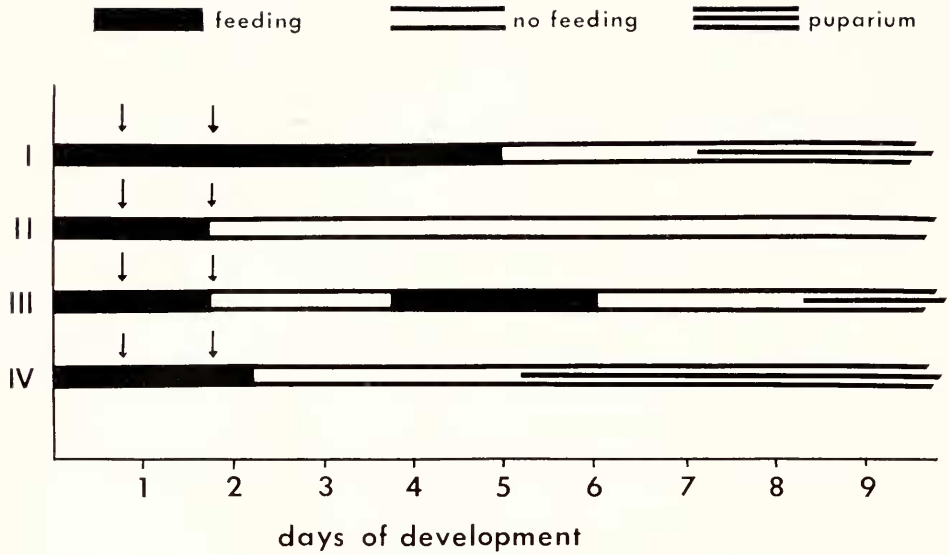

FIGURE 1. Diagrammatic illustration of the relationships between feeding and development in larvae of *Calliphora vomitoria*; I, normal development; II, non-developing larvae starved since the last larval ecdysis; III, larvae temporarily starved for determined period in the 3rd instar; IV, larvae allowed to feed only 12 hours after the last larval ecdysis. The arrows indicate larval ecdyses.

2. *Combined effects of starvation and high doses of ecdysterone*

Small amounts of ecdysone or ecdysterone are known to stimulate the metamorphosis processes. Relatively large doses cause, as a rule, precocious ecdysis with the form of the body corresponding to the degree of developmental determination at the moment of injection. Therefore, we applied large doses of ecdysterone to larvae of the third instar before the mechanisms which determine metamorphosis were activated. From our previous observations we calculated that this period must occur at the very beginning of the last larval instar or in

TABLE I

Effects of feeding on the action of high doses of ecdysterone (20 μg per larva) in the 3rd larval instar of Calliphora

Feeding period and ecdysterone injection (hr after ecdysis)	No. of larvae	Effects 24 hours after injection			
		Supernumerary larvae	Intermediates	Puparia	Dead
0	17	12	0	0	5
3	21	15	1	0	5
6	27	16	8	0	4
12	26	0	14	4*	7
24	25	0	0	22	2

* Incompletely contracted.

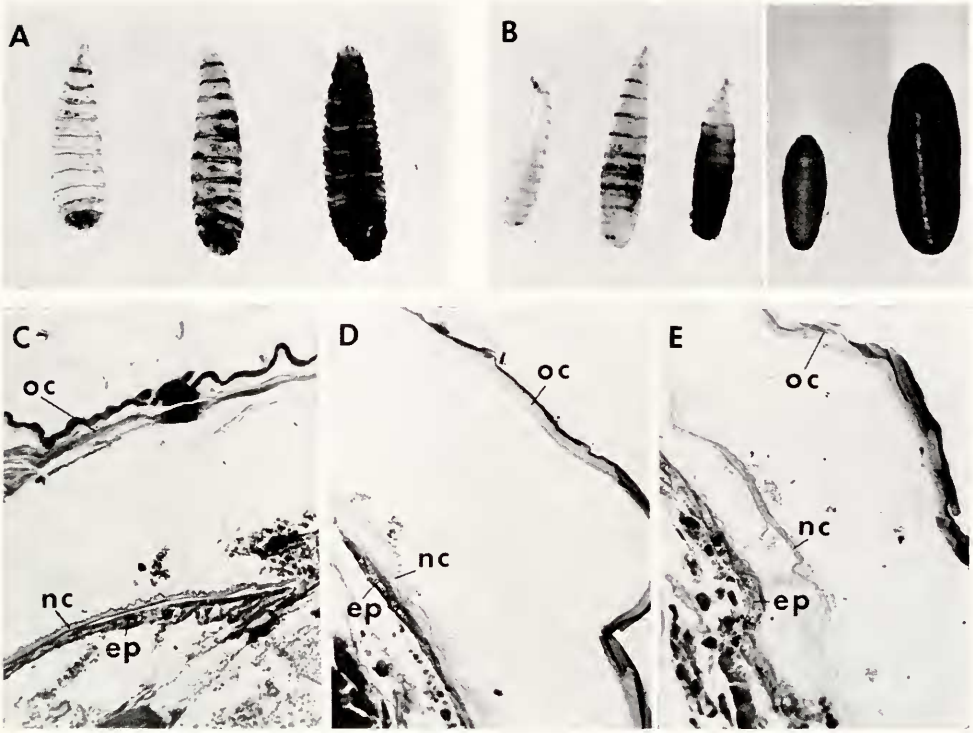

FIGURE 2. Supernumerary larvae and larvae-puparium intermediates in *Sarcophaga* and *Calliphora;* (A) larva-puparium intermediates of *Sarcophaga* with different degree of pupariation; (B) supernumerary larva, two larva-puparium intermediates, dwarf puparium and normal puparium in *Calliphora*; (C) section through integument of *Sarcophaga* larvae after ecdysterone injections showing the old 3rd instar cuticle and the new extra-larval cuticle; (D) the same in *Calliphora*; (E) similar section in *Calliphora* showing the detachment of the extra-larval cuticle from the epidermal cells. Abbreviations are: oc—old cuticle of the 3rd instar larva, nc—new extra-larval cuticle, ep—epidermal cells.

starved last instar larvae. As a matter of fact, injections performed at these stages induced precocious molt and secretion of an extra-larval cuticle (Table I). The supernumerary larvae produced in this way were of the same size as newly ecdysed third instar larvae. The new cuticle was apparent approximately 24 hours after the injection, but the larvae were unable to shed the non-sclerotized and incompletely digested old larval cuticle. The newly formed extra-larval cuticle had perfect larval characteristics such as a set of stigmatic plates on the hind spiracles, tranverse bands of segmental spinules, *etc.* (Fig. 2).

Similar injections of ecdysterone into larvae which received a subcritical amount of feeding (3 hours) also produced supernumerary larval instars. By increasing the time of feeding we obtained supernumerary larvae and intermediates with a wide range of mosaic puparied spots in the old cuticle. With even more feeding we obtained intermediates possessing more of the puparied spots in the old cuticle and no supernumerary larvae. And, finally, after 24 hours of feeding more or less normal puparia were formed (Table I). These results

TABLE II

Effects of high and low doses of ecdysterone on starved (non-developing) 3rd instar larvae of Calliphora

Ecdysterone (in μg per larva) injected at		Effects observed 24 hours after the last injection		
0 hours after ecdysis	72 hours after ecdysis	Formation of new cuticle	Developmental stage achieved	Degree of pupariation*
20	0	Yes	Supernumerary larva	0%
0	20	Yes	Supernumerary larva	0%
5	0	No	Permanent larva	
0	5	No	Permanent larva	
1	20	Yes	Larva-puparium intermediate	10%
2	20	Yes	Larva-puparium intermediate	30%
5	20	Yes	Larva-puparium intermediate	20%
1	2	Yes	Larva-puparium intermediate	30%
2	2	Yes	Larva-puparium intermediate	50%
5	2	Yes	Larva-puparium intermediate	70%

* Based on the estimated tanned area of the old cuticle.

with high doses of ecdysterone demonstrate that when the larvae are able to feed, the initiation of processes of metamorphosis and the associated loss of ability of the epidermal cells to form the larval patterns are taking place in the 24 hour interval after the last larval ecdysis.

In further experiments we tried to find out to what extent was the decision-making mechanism which would either maintain the *status quo* or commit the cells and tissues to metamorphosis dependent on feeding. In these experiments larvae starved from the moment of ecdysis were injected with high doses of ecdysterone after 24 or 72 hours of starvation. In all cases we got only perfect supernumerary larvae (Table II). Evidently, the whole endogenous mechanism for the determination of metamorphosis remains inhibited in the absence of food.

The individuals destined to form supernumerary larvae or intermediates underwent a precocious molt characterized by apolysis, more or less profound digestion of the old cuticle, and the formation of a thin new cuticle (Fig. 2 C, D). All the larvae treated with high doses of ecdysterone were unable to feed; they had limited ability to move, and were unable to shed the old cuticle. They died after 2 to 3 days. When they were dissected we got an impression that the supernumerary larva showed apolysis of its cuticle. The latter in some cases could be removed and underneath remained a delicate integumentary layer. This was confirmed on some histological sections. Thus, as seen in Figure 2 E, the extra-larval cuticle is detached from the epidermal cells and partially digested. In the case of larval-puparium intermediates the old partially pupariated cuticle was always separated from the epidermis. The newly formed cuticle was never pupariated.

3. *Effects of small doses of ecdysterone*

After we realized that the endogenous determination process was suppressed in starved larvae, we examined the effects of small doses of ecdysterone. While

doses of 20 μg per larva always induced precocious ecdysis, doses of 5 μg and less did not have this effect.

The data in Table II demonstrate that small doses of ecdysterone injected after ecdysis, combined with large doses injected 3 days later, produced intermediates with various degrees of tanning in the old cuticle. It is interesting to note that as little as 1 μg of ecdysterone injected after ecdysis affects the metamorphosis determination process in certain areas of epidermis; it results ultimately in tanning of the old cuticle above the affected epidermal cells. Combinations of two small doses, one after ecdysis and the other 3 days later also led to the larva-puparium intermediates, although the total amount of ecdysterone injected was far below the amount necessary for precocious ecdysis. As seen at the bottom of Table II, the greater the dose of ecdysterone injected immediately after ecdysis, the more extensive was the area of tanned cuticle of the puparial type. Thus, by administering repeated low doses of ecdysterone to these starved larvae, we succeeded in partially imitating the endogenous mechanism regulating the initial processes of metamorphosis in these flies.

Discussion

Our results indicate that the number of larval instars in cyclorrhaphous flies is under the control of the neuroendocrine system. The hormonal mechanism synchronizing development with the availability of food has been known for a long time in diverse insect species (Thomsen, 1952; Johansson, 1958; Strangways-Dixon, 1961; Engelmann, 1968).

We have found that the "gene-set switching mechanism" for metamorphosis occurs in these flies very early after the last larval ecdysis when enough food is available. The perfect supernumerary larvae can be produced only by excessive amounts of ecdysterone and only when this is administered just after the ecdysis or injected into starved third instar larvae. With feeding third instar larvae older than 24 hours it was impossible to obtain anything but puparia. In addition, small amounts of ecdysterone never caused perfect supernumerary larvae, but in combination with repeated administration of ecdysterone the larvae formed more or less advanced larva-puparium intermediates. This confirms the qualitative difference between the effects of excessive and small amounts of ecdysones (Williams, 1968). We conclude that the excessive amounts of ecdysterone cause immediate realization of the existing genetical information. By contrast, the small amounts of the hormone cause progressive switching on of the gene-sets responsible for the metamorphosis development.

During the normal development the third instar larvae most likely maintain a low level of endogenous ecdysones conditioning the programming of processes leading to puparium formation. In some instances, the hormone may temporarily disappear and the puparium formation is consequently delayed, as is the case of *Sarcophaga* larvae kept in contact with water (Ohtaki, Milkman and Williams, 1968; Zdarek and Fraenkel, 1970) or diapausing larvae of *Lucilia* (Fraser and Smith, 1963). The fact that so many investigators applying ecdysones to final larval instars of higher Diptera failed to obtain the supernumerary larval instars may be explained in the following way. Most investigators used advanced third

instars where the developmental program for metamorphosis had already been determined. Secondly, they may not have expected that the required amounts of the hormone would be so extremely high. To get a positive result in the puparium assay on *Calliphora* larvae, one needs to inject approximately 0.01 μg of ecdysterone per ligated abdomen. However, in order to get supernumerary ecdysis in starved larvae of *Calliphora* (which is about 10 times smaller in weight) we need at least 10 μg of the hormone, *i.e.*, 10,000 times more per unit of weight.

We are greatly indebted to Prof. C. M. Williams of Harvard University for helpful criticism and corrections of the English.

Summary

1. The developmental program for metamorphosis is determined in third instar larvae of *Calliphora* and *Sarcophaga* very early after ecdysis. The process is activated by feeding and can be delayed for many days by starvation.

2. High doses of ecdysterone (20 μg per larva) injected just after ecdysis or in starved larvae cause a precocious molt and the formation of supernumerary larvae. The same treatment of larvae a few hours after ecdysis leads to larva-puparium intermediates displaying the mosaic of tanned areas in the old cuticle. Larvae older than 24 hours after ecdysis responded only by forming small puparia.

3. Small doses of ecdysterone (less than 5 μg per larva) do not cause a complete molt but activate the "gene-set switching mechanism" for programming of the metamorphosis development. The degree of such a covert effect can be revealed by repeated injections of ecdysterone which lead to formation of more or less advanced larva-puparium intermediates.

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